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(54) Title: METHOD FOR SELECTIVE METHIONINE STARVATION OF MALIGNANT CELLS IN MAMMALS		
(57) Abstract <p>An improved method for chemotherapy of mammalian malignant cells which have an absolute requirement for methionine but lack methylthioadenosine phosphorylase (MTase). The method comprises detection of MTase negative cells in a mammal, administration of methionine γ-lyase in sufficient amounts to reduce the volume of MTase negative cells in the mammal, and co-administration of methylthioadenosine in amounts sufficient to ensure the continued availability of methionine to the mammal's non-malignant cells. Means for detection of MTase negative cells are provided. Means for production and use of recombinant chemotherapeutic agents are also provided.</p> <p style="text-align: right;">BEST AVAILABLE COPY</p>		

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METHOD FOR SELECTIVE METHIONINE
STARVATION OF MALIGNANT CELLS
IN MAMMALS

5 BACKGROUND OF THE INVENTION

1. Field of the Invention

This invention relates to a method for the selective destruction of malignant cells in mammals based on metabolic differences between those cells and non-
10 malignant (i.e., "normal") cells. More specifically, it relates to starvation of malignant cells which lack the enzyme necessary to convert methylthioadenosine to methionine by degrading plasma methionine and homocysteine.

15

2. History of the Invention

The amino acid methionine (MET) is necessary for the growth of normal and malignant cells. In certain malignant cells this requirement is absolute, i.e.,
20 without an adequate supply of MET, the cells die.

In mammalian cells, MET is obtained from three sources. It can be obtained in the diet, or through biochemical synthesis of MET from L-homocysteine (homocysteine) or methylthioadenosine (MTA) (a product of
25 the polyamine biosynthetic pathway). In the latter case, MTA is converted to MET by methylthioadenosine phosphorylase (MTase).

In the past decade, researchers have identified many malignant cell lines which lack MTase and cannot,
30 therefore, convert MTA to MET. For example, Katamari, et al., *Proc. Nat'l Acad. Sci. USA*, 78:1219-1223 (1981) reported that 23% of 3 human malignant tumor cell lines lacked detectable MTase, while MTase activity was present in each of 16 non-malignant cell lines studied. MTase
35 negative cells principally fulfill their requirement for MET through conversion of homocysteine. However, when

homocysteine is not available, the cells will generally die.

L-methionine-L-deamino-γ-mercaptomethane lyase (ED 4.4.1.11; METase) is known to degrade not only MET but also homocysteine. Theoretically, therefore, one could starve malignant cells which lack MTase (i.e., MTase negative cells) by degrading plasma MET and homocysteine with METase. Normal MTase positive cells would be expected to fulfill their requirement for MET by the continued conversion of MTA to MET.

A rudimentary version of this approach was first proposed in 1972 by Kreis in *Cancer Treat. Rpts.*, 63: 1069-1072 (1972). Using 11 malignant cell lines in MET-free cultures, Kreis was able to inhibit the growth of certain of the malignant cells by applying METase to the cultures. Kreis also observed that 2 normal cell lines were partly "rescued" from the effects of MET starvation when homocysteine was added to the cultures. However, while these *in vitro* studies were encouraging, several obstacles were described by Kreis as being in the way of a successful *in vivo* use of METase in chemotherapy, including the unavailability of means to ensure the survival of normal cells *in vivo*, the potential immunogenicity of purified or partially purified enzyme, and the need for the enzyme to be resistant to degradation by proteolytic enzymes *in vivo* (Kries, *Chemotherapy* (Muggia, FM, ed., The Hague, Boston, and London: Martinus-Nijhoff, 1983), pp. 219-248).

Another obstacle to the development of a successful approach to MET starvation of malignant cells has been the need to identify which malignancies are suitable targets for the therapy; i.e., which malignancies are MTase negative. To that end, an assay was developed which predicts whether a malignancy is MTase negative by determining whether any catalytic activity is present in a cell culture (Seidenfeld, et al., *Biochem. Biophys. Res. Commun.*, 95:1861-1866, 1980). However, because of

the commercial unavailability of the radiochemical substrate required for the activity assay, its use in routine evaluations is not presently feasible. Moreover, the activity assay does not account for the catalytic
5 lability of MTase *in vitro* by detecting whether any of the enzyme is present in the cell culture regardless of whether it is catalytically active at the time that the assay is performed.

This limitation of the activity assay could be
10 avoided by the development of an immunoassay which is sufficiently sensitive to detect relatively minute quantities of enzyme. However, the purification of the MTase enzyme from natural sources to develop antibodies for use in immunological detection of MTase has proven to
15 be a laborious process which produces relatively poor yields (Rangione, et al., *J. Biol. Chem.*, 261:12324-12329, 1986).

Even if adequate means were developed to detect MTase negative cells, production of an adequate supply of
20 METase from natural sources has been as difficult as the production of MTase. Production of METase by means other than purification of the native enzyme has not yet been achieved, in part because the gene for METase has (to date) been only partially sequenced (Nakayama, et al.,
25 *Biochem*, 27:1587-1591, 1988).

For all of these reasons, an effective approach to *in vivo* MET starvation of MTase malignant cells has remained elusive. The present invention addresses this need.

30

SUMMARY OF THE INVENTION

In combination with means for detecting MTase negative cells, the invention comprises an improved method for the selective starvation of MTase negative
35 cells. According to the method, a malignancy which has been determined to be MTase negative is treated with a therapeutically effective amount of METase, preferably

recombinant METase, and most preferably recombinant METase conjugated to polyethylene glycol or an equivalent molecule. More specifically, METase is administered to a mammal (preferably a human) in a dosage which will its
5 lower plasma MET levels to an extent sufficient to starve MTase negative cells of methionine (which will generally occur at about \leq 10% of the pre-therapy level of methionine). Normal (MTase positive) cells are supplied with MET through the substantially contemporaneous
10 administration of MTA.

The invention also comprises in part a method for detecting MTase negative cells in a malignancy. More specifically, it comprises in one aspect the production of anti-MTase antibodies (including monoclonal
15 antibodies) and their use in an immunoassay for MTase. In another aspect, it comprises detection of the presence of the gene which encodes MTase by use of an assay based on nucleic acid amplification techniques, in particular the polymerase chain reaction (PCR).

20 The invention also comprises recombinant METase developed from the isolation and cloning of the gene encoding METase, thus enabling the production of substantial quantities of METase for use in the methods of the invention.

25

BRIEF DESCRIPTION OF THE DRAWINGS

FIGURE 1 is a schematic of the metabolic pathway for polyamine synthesis and reduction of MTA by MTase.

FIGURE 2 is a comparison of MTase positive and MTase
30 negative human and non-human cell lines detected by immunoblot analysis.

FIGURE 3 is a comparison of MTase positive and MTase negative human cell lines and primary tumors detected by immunoblot analysis.

35 FIGURE 4 is a comparison of the growth experienced by MTase negative human cells treated with METase versus those grown in a methionine rich environment.

DETAILED DESCRIPTION OF THE INVENTION

I. METHOD FOR DETECTION OF MTase NEGATIVE CELLS.

FIGURE 1 schematically depicts the metabolic pathways for *in vivo* synthesis of MET from MTA and degradation of MET by METase. As indicated above, to gain the full benefits of a methionine starvation cancer therapy, MTase negative cells must be detected in the target malignancy. To that end, alternative means of detecting MTase which are suitable for use in the methods of the invention are described below.

A. Immunoassay for MTase.

1. Production of Antigenic MTase and MTase Peptides.

Antibodies which are specific for MTase are produced by immunization of a non-human with antigenic MTase or MTase peptides. Generally, the antigenic MTase peptides may be isolated and purified from mammalian tissue according to the method described by Ragnione, et al., *J. Biol. Chem.*, 265: 6241-6246 (1990). An example illustrating the practice of this method is provided in the Examples below. For reference, the amino acid sequence for full-length MTA is included herein as SEQ. ID. NO. 1.

2. Immunization with Antigenic MTase Peptides to Produce Anti-MTase Antibodies

Once antigenic MTase or MTase peptides are obtained, antibodies to the immunizing peptide are produced by introducing peptide into a mammal (such as a rabbit, mouse or rat). For purposes of illustration, the amino acid sequences of two antigenic MTA peptides are provided in the Sequence Listing appended hereto as SEQ ID. Nos. 2 and 3. Antibodies produced by rabbits immunized with these peptides showed a 50% maximal response to purified MTA at, respectively, a 1:1500 and a 1:4000 dilution.

A multiple injection immunization protocol is preferred for use in immunizing animals with the

antigenic MTase peptides (see, e.g., Langone, et al., eds., "Production of Antisera with Small Doses of Immunogen: Multiple Intradermal Injections", *Methods of Enzymology* (Acad. Press, 1981)). For example, a good
5 antibody response can be obtained in rabbits by intradermal injection of 1 mg of the antigenic MTase peptide emulsified in Complete Freund's Adjuvant followed several weeks later by one or more boosts of the same antigen in Incomplete Freund's Adjuvant.

10 If desired, the immunizing peptide may be coupled to a carrier protein by conjugation using techniques which are well-known in the art. Such commonly used carriers which are chemically coupled to the peptide include keyhole limpet hemocyanin (KLH), thyroglobulin, bovine
15 serum albumin (BSA), and tetanus toxoid. The coupled peptide is then used to immunize the animal (e.g. a mouse or a rabbit). Because MTase is presently believed to be conserved among mammalian species, use of a carrier protein to enhance the immunogenicity of MTase proteins
20 is preferred.

Polyclonal antibodies produced by the immunized animals can be further purified, for example, by binding to and elution from a matrix to which the peptide to which the antibodies were raised is bound. Those of
25 skill in the art will know of various techniques common in the immunology arts for purification and/or concentration of polyclonal antibodies, as well as monoclonal antibodies (see, for example, Coligan, et al., Unit 9, *Current Protocols in Immunology*, Wiley Interscience,
30 1991).

For their specificity and ease of production, monoclonal antibodies are preferred for use in detecting MTase negative cells. For preparation of monoclonal antibodies, immunization of a mouse or rat is preferred.
35 The term "antibody" as used in this invention is meant also to include intact molecules as well as fragments thereof, such as for example, Fab and F(ab')₂, which are

capable of binding the epitopic determinant. Also, in this context, the term "mAb's of the invention" refers to monoclonal antibodies with specificity for MTase.

The general method used for production of hybridomas secreting monoclonal antibodies ("mAb's") is well known (Kohler and Milstein, *Nature*, 256:495, 1975). Briefly, as described by Kohler and Milstein, the technique comprised isolation of lymphocytes from regional draining lymph nodes of five separate cancer patients with either melanoma, teratocarcinoma or cancer of the cervix, glioma or lung. The lymphocytes were obtained from surgical specimens, pooled, and then fused with SHFP-1. Hybridomas were screened for production of antibody which bound to cancer cell lines. An equivalent technique can be used to produce and identify mAb's with specificity for MTase.

Confirmation of MTase specificity among mAbs of the invention can be accomplished using relatively routine screening techniques (such as the enzyme-linked immunosorbent assay, or "ELISA") to determine the elementary reaction pattern of the mAb of interest.

It is also possible to evaluate an mAb to determine whether it has the same specificity as a mAb of the invention without undue experimentation by determining whether the mAb being tested prevents a mAb of the invention from binding to MTase. If the mAb being tested competes with the mAb of the invention, as shown by a decrease in binding by the mAb of the invention, then it is likely that the two monoclonal antibodies bind to the same or a closely related epitope.

Still another way to determine whether a mAb has the specificity of a mAb of the invention is to pre-incubate the mAb of the invention with an antigen with which it is normally reactive, and determine if the mAb being tested is inhibited in its ability to bind the antigen. If the mAb being tested is inhibited then, in all likelihood, it

has the same, or a closely related, epitopic specificity as the mAb of the invention.

3. Immunoassay Protocol for Detection of MTase Negative Cells.

5 Once suitable antibodies are obtained as described above, they are used to detect MTase in a malignancy. An example of an immunoassay suitable for this purpose (i.e., an immunoblot method) is described further in Example I below. However, those skilled in the
10 immunological arts will recognize that MTase may be detected using the antibodies described above in other immunoassay formats, in either liquid or solid phase (when bound to a carrier).

Detection of MTase using anti-MTase antibodies can
15 be done utilizing immunoassays which are run in either the forward, reverse, or simultaneous modes, including immunohistochemical assays on physiological samples. Suitable immunoassay protocols include competitive and non-competitive protocols performed in either a direct or
20 indirect format. Examples of such immunoassays are the radioimmunoassay (RIA) and the sandwich (immunometric) assay. Those of skill in the art will know, or can readily discern, other immunoassay formats without undue experimentation.

25 In addition, the antibodies utilized in the immunoassays may be detectably labelled. A label is a substance which can be covalently attached to or firmly associated with a nucleic acid probe which will result in the ability to detect the probe. For example, a label
30 may be radioisotope, an enzyme substrate or inhibitor, an enzyme, a radiopaque substance (including colloidal metals), a fluoresceors, a chemiluminescent molecule, liposomes containing any of the above labels, or a specific binding pair member. A suitable label will not
35 lose the quality responsible for detectability during amplification.

Those skilled in the diagnostic art will be familiar with suitable detectable labels for use in in vitro detection assays. For example, suitable radioisotopes include ^3H , ^{125}I , ^{131}I , ^{32}P , ^{14}C , ^{35}S . Amplified fragments
5 labeled by means of a radioisotope may be detected directly by gamma counter or by densitometry of autoradiographs, by Southern blotting of the amplified fragments combined with densitometry. Examples of suitable chemiluminescent molecules are acridines or
10 luminol. Target sequences hybridized with probes derivatized with acridium ester are protected from hydrolysis by intercalation. Examples of suitable fluorescers are fluorescein, phycobiliprotein, rare earth chelates, dansyl or rhodamine.

15 Examples of suitable enzyme substrates or inhibitors are compounds which will specifically bind to horseradish peroxidase, glucose oxidase, glucose-6-phosphate dehydrogenase, β -galactosidase, pyruvate kinase or alkaline phosphatase acetylcholinesterase. Examples of
20 radiopaque substance are colloidal gold or magnetic particles.

A specific binding pair comprises two different molecules, wherein one of the molecules has an area on its surface or in a cavity which specifically binds to a
25 particular spatial and polar organization of another molecule. The members of the specific binding pair are often referred to as a ligand and receptor or ligand and anti-ligand. For example, if the receptor is an antibody the ligand is the corresponding antigen. Other specific
30 binding pairs include hormone-receptor pairs, enzyme substrate pairs, biotin-avidin pairs and glycoprotein-receptor pairs. Included are fragments and portions of specific binding pairs which retain binding specificity, such as fragments of immunoglobulines, including Fab
35 fragments and the like. The antibodies can be either monoclonal or polyclonal. If a member of a specific

binding pair is used as a label, the preferred separation procedure will involve affinity chromatography.

The antibodies may also be bound to a carrier. Examples of well-known carriers include glass, polystyrene, polypropylene, polyethylene, dextran, nylon, amyloses, natural and modified celluloses, polyacrylamides, agaroses and magnetite. The nature of the carrier can be either soluble or insoluble for purposes of the invention. Those skilled in the art will know of other suitable carriers for binding antibodies, or will be able to ascertain such, using routine experimentation.

B. Detection of MTase Negative Cells Using a PCR-based Assay.

For the relative ease and speed of detection provided by immunoassay using the MTase-specific antibodies described herein, the immunoassay is the preferred means for detection of MTase-negative cells. However, those skilled in the art will also recognize that other detection means to detect the presence of MTase negative cells in a malignancy may be used. For example, using the nucleic acid sequence description in SEQ. ID NO 1, one of skill in the art could construct oligonucleotide probes which would hybridize to MTase DNA present in a cell sample. Conversely, because it is believed that MTase deficiency results from the genomic deletion of the gene which would encode the MTase protein, it can be assumed that if no gene encoding MTase is detected in a cell sample that the cells are MTase negative.

A detailed description of a protocol for the amplification and detection of the MTase gene is provided in co-pending U.S. patent application Serial No. 08/176,855, filed December 29, 1993. The disclosure of co-pending application No. 08/176,855 pertaining to this protocol is incorporated herein by this reference.

C. MTase Negative Candidates for MTase Starvation Therapy

A malignancy which is a candidate for the therapy of the invention (i.e., MET starvation therapy) is one in which the MTase protein, whether catalytically active or catalytically inactive, is not detectably present. In all malignant cell lines studied to date, MTase negativity (if present) is a consistent trait throughout the cell population. In other words, if some cells of a malignancy are MTase negative, it can be expected that all cells of the malignancy will be MTase negative. This is consistent with the present belief in the art that MTase deficiency is the result of a gene deletion rather than a mutation. The homogeneity of a malignancy for MTase negativity should significantly enhance the efficacy of MET starvation as a cancer therapy in comparison to therapies directed to heterogeneous traits, such as tumor antigens targeted in monoclonal antibody therapy. However, it is sufficient for purposes of the invention that the malignancy be "substantially deficient" in MTase; i.e., that they contain no detectable quantities of MTase protein.

Human malignancies which are presently believed to be substantially deficient in MTase include:

25

20 U-87MG
U-138MG
Hs683

25 Primary brain tumors: Immunoassay♦
Astrocytoma
Glioblastoma multiforme
Oligostrocytoma

30 Lymphomas and leukemias: Immunoassay♦
CEM (acute lymphocytic leukemia)
K-T-1 (acute lymphocytic leukemia)
NALL-1 (acute lymphocytic leukemia)
35 K562 (chronic myelogenous leukemia)
DHL-9 (malignant lymphoma)
HSB2 (acute lymphocytic leukemia)

40 Other:
Walker 256 sarcomasarcoma C l i n i c a l
evidence**
Jurkat Immunoassay***
K562 Immunoassay***
45 Capan-1 (adenosarcoma of pancreas) Immunoassay****

LEGEND:

*obtained from the American Type Culture Collection,
Rockville, MD.

50 **reported by Kries, et al., *Cancer Res.*, 33:1866-
1869 (1973)

***reported by Rangione, et al., *Biochem. J.* 281:533-538 (1992)

****reported by Kries, et al., *Cancer Trmt. Rpts.*, 63:1069-1072 (1979)

- 5 ♦MTase deficiency in all other malignancies was detected and reported by Nobori, et al., in *Cancer Res.* 53:1098-1101 (1993) and in *Cancer Res.* 51:3193-3197 (1991).

10 Using the detection techniques described herein, those skilled in the art will be able to detect MTase deficiency in other malignancies without undue experimentation.

II. MET STARVATION THERAPY

15 A. Production of METase

For use in the methods of the invention, sources of both MTA and METase are required. Means for obtaining MTA are described *supra*. For use in the methods of the invention, METase has been purified from microorganisms
20 including *Trichomonas vaginalis* (Lockwood, et al., *J. Biochem.* 279:675-682, 1991), *Clostridium sporogenes* (*see*, e.g., Kries, et al., *supra* at 1867; EC4.4.1.11), and *Pseudomonas putida* (Nakayama, et al., *Biochem.*, 27:1587-1591, 1988).

25 Using a cDNA library constructed from *P. putida*, the full-length nucleotide sequence for METase has been identified and is contained in the Sequence Listing appended hereto as SEQ. ID. No. 4; the amino acid sequence is contained in SEQ ID NO. 5.

30 With this information, METase can be readily synthesized or expressed from a DNA clone using well-known techniques as described above with respect to MTase. A detailed example of how METase can be cloned and expressed in *E. coli* is provided further below in
35 Examples II and III.

While purified, partially purified, synthesized or recombinant METase may be used in the therapeutic method of the invention, the latter is preferred for its ease of production and relatively low immunogenicity. The immunogenicity of the enzyme can be, and preferably will be, further reduced by coupling it to polyethylene glycol (PEG) or an equivalent, biologically compatible molecule. Coupling to PEG can also be expected to reduce the half-life of the METase conjugate *in vivo*.

10 The PEG-METase conjugate can be formed by covalent attachment of PEG to the enzyme as described with respect to L-asparagine (see, e.g., Benedich, et al., *Clin. Exp. Immunol.* 48:273-278, 1982). Methods for coupling PEG to proteins are well-known in the art and will not, therefore, be described further in detail here. Based on results observed in human clinical trials for treatment of non-hodgkins lymphoma with L-asparaginase coupled to PEG, coupling of METase to PEG would not be expected to significantly reduce its activity *in vivo* (see, *re in vivo* results obtained with PEG-L-asparaginase, Muss, et al., *Invest. New Drugs*, 8:125-130 (1990)). Those skilled in the art will recognize, however, that other means for extending the half-life of proteins *in vivo* are known and may be suitable for use with METase including, but not limited to, glycosylation and succinylation.

B. Therapeutic Methods.

Malignancies which are substantially deficient in MTase will be treated according to the invention in part by administration of METase. Preferably, these malignancies will be those which can be treated by regional chemotherapy; i.e., where the malignancy is localized and contained in an area of the body which is accessible by intra-arterial infusion or by introduction through topical, transdermal or equivalent routes for administration of the METase directly to the locus of the malignancy. Examples of malignancies which are susceptible to regional chemotherapy are melanomas,

ovarian cancer (via a peritoneal catheter) and bladder cancer (via a urethral catheter). Other malignancies which, if MTase negative, may be treated by regional chemotherapy according to the invention will be known by those skilled in oncology.

It will be appreciated by those skilled in the art that the therapeutic compositions of the invention may also be administered systemically. However, the dosages would have to be adjusted to compensate for clearance of the compositions and potential toxicity to normal cells. In particular, clinical evidence of methionine starvation of normal cells would have to be monitored closely and compensated for, if necessary, by administration of additional quantities of MTA.

Malignancies which are substantially deficient in MTase will preferably be treated according to the invention as follows.

METase will be administered to a mammal (preferably a human) parenterally, with the preferred route of administration being intra-arterial infusion. The METase will be administered in a pharmaceutically acceptable carrier, which may include sterile aqueous or non-aqueous solutions, suspensions, and emulsions. Examples of non-aqueous solvents are propylene glycol, polyethylene glycol, vegetable oils such as olive oil, and injectable organic esters such as ethyl oleate. Aqueous carriers include water, alcoholic/aqueous solutions, emulsions or suspensions, including saline and buffered media. As noted above, the METase will preferably be conjugated to PEG to reduce its immunogenicity.

Parenteral vehicles include sodium chloride solution, Ringer's dextrose, dextrose and sodium chloride, lactated Ringer's or fixed oils. Intravenous vehicles include fluid and nutrient replenishers, electrolyte replenishers (such as those based on Ringer's dextrose), and the like. Preservatives and other additives may also be present such as, for example,

antimicrobials, antioxidants, chelating agents, and inert gases and the like.

Dosages of the METase can vary from about 10 units/m² to 20,000 units/m², preferably from about 5000 to 6000 units/m², (or lower when administered by intra-arterial infusion) in one or more dose administrations weekly, for one or several days. METase can generally be expected to be cleared by the mammal in about 24 hours after its administration; with use of means to extend the half-life of the enzyme such as PEG conjugation, this half-life may be extended by several hours to several days. The mammal's plasma methionine levels should, therefore, be monitored and additional doses of METase administered as necessary to achieve a therapeutically significant reduction of the mammal's plasma methionine concentration. This will be a reduction sufficient to induce a detectable decrease in the volume of MTase negative cells; i.e., a decrease in the volume of malignant cells or tumor load in the mammal. A dosage which achieves this result will be considered a "therapeutically effective" dosage. Based on *in vivo* studies in rodents using partially purified METase, a therapeutically effective dosage can be generally expected to be one which reduces the plasma methionine level in the patient to about $\leq 10\%$ of its pre-therapy level.

Plasma methionine levels (and changes therein) can be monitored by periodic (and preferably daily) *in vitro* assays of blood samples drawn from the patient receiving the METase throughout the course of its administration. Generally, based on the studies done in rodents, it can be expected that plasma methionine levels will be lowered to $\leq 10\%$ of their pretherapy levels within about an hour of the administration of METase. Assays for plasma methionine are well-known in the art; for example, the concentration of methionine in a blood sample can be determined using the method for gas-liquid chromatography of esterified amino acids (*n*-butyl ester) is described in

Roach, et al., *J.Chromatog.* 44:269-278 (1969). Other equivalent procedures to detect methionine in plasma will be known to or easily identified by those of ordinary skill in the art.

5 It should be noted that METase cannot degrade intracellular methionine. Therefore, with an adequate supply of MTA for the formation of intracellular methionine, MTase-positive cells will generally be able to survive the reduction of exogenous methionine by
10 METase. However, without a supply of exogenous methionine (or the L-homocysteine substrate for methionine which is also degraded by METase), MTase-negative cells with an absolute requirement for methionine will generally not survive the loss of plasma
15 methionine.

The efficacy of the therapy may be confirmed and monitored by any clinical evidence indicative of a reduction in the cellular volume of the malignancy (determined by means well known in the art) and/or
20 periodic detection of the MTase-negative cell volume in the malignancy using the detection means described herein. Based on clinical data regarding the use of L-asparaginase therapy in humans, it can be expected that the toxicity of the METase therapy will be fairly low and
25 may consist primarily of allergic reactions treatable by means well known to those skilled in the clinical art, such as administration of epinephrine.

Therefore, MTA will be administered to the mammal substantially concurrently with METase. Preferably, the
30 MTA and METase will be administered at the same time. Because MTA will not act as a substrate for METase, the two may be combined together in a pharmaceutically acceptable carrier. Alternatively, the MTA may be administered within about 24 hours of the administration
35 of the METase (and preferably sooner) to "rescue" the MTase positive cells whose endogenous supply of methionine is becoming exhausted.

The dose of MTA needed to rescue normal cells will vary depending on a number of clinical factors, including the location of the malignancy, the volume of MTase negative cells in the malignancy, the length of METase therapy and the availability to the patient of dietary MET. Generally, however, the MTA will be administered in dosages sufficient to maintain a plasma methionine level of about 1-10 μ M.

The invention having been fully described, examples illustrating its practice are set forth below. These examples should not, however, be considered to limit the scope of the invention, which is defined by the appended claims.

In the examples, the abbreviation "min." refers to minutes, "hrs" and "h" refer to hours, and measurement units (such as "ml") are referred to by standard abbreviations.

EXAMPLE I

IMMUNOASSAY FOR MTase

20 A. Production of MTase Antibodies

MTase was purified from bovine liver as described by Rangione, et al., supra. Several tryptic peptides from the isolated enzyme were sequenced using conventional techniques. Based upon the sequences obtained, peptides 25 40 (18 amino acids long; see SEQ. I.D. No. 2) and 51 (14 amino acids long; see SEQ. I.D. No. 3) were synthesized by a modification of the well-known Merrifield solid-phase method (see, e.g., Chen, et al., *Proc. Nat'l Acad. Sci. USA*, 81:1784-1788, 1984). All peptides contained a 30 cysteine residue at the carboxy terminus to facilitate chemical coupling to the carrier protein, KLH, with m-maleimidobenzoyl-N-hydroxysuccinimide ester.

New Zealand white rabbits (two rabbits per peptide) were immunized on a bimonthly basis with the peptide-KLH 35 conjugates. The initial injections contained 1 mg of synthetic peptide-KLH conjugate emulsified in Freund's complete adjuvant. Booster injections had 1 mg of

antigen in incomplete Freund's adjuvant. After 3-4 injections, sera were partially purified with 50% saturated ammonium sulfate and were screened for anti-peptide and anti-MTase reactivities by ELISA.

5 More specifically, microtiter plates were precoated with peptides or MTase at 10 µg/ml in BBS (0.2 M sodium borate-0.15 M NaCl, pH 8.5) overnight at 4°C. The plates were washed once in BBS containing 0.05% Tween 20 and then were incubated for 4 hours with BBS containing 1%
10 bovine serum albumin to block nonspecific binding sites. Several dilutions of a control serum or peptide-induced antisera were then applied in 0.1-ml aliquots and incubated overnight. The plates were washed twice with BBS containing 0.05% Tween 20, and then exposed for 1
15 hour to alkaline phosphatase-labeled goat F(ab')₂ anti-rabbit immunoglobulin (Jackson Laboratories, Inc., West Grove, PA) at a dilution of 1:1000 in BBS. After the plates were washed, 0.2 ml of 0.1 M p-nitrophenyl phosphate disodium in 0.1 M NaHCO₃, pH 9.0, was added to
20 each well. The absorption at 405 nm was measured 30 minutes later.

B. Protocol for Immunoblot Analysis for Immunoreactive MTase

Several human cell lines and tumor biopsies were
25 evaluated for the presence of MTase-negative cells (see, re the MTase-negative cells, Table I, items marked "immunoassay"). Other sample which tested MTase-positive were BV-173 (a chronic myelogenous leukemia, "CML"), Molt-16 (an acute lymphocytic leukemia, "ALL"), Molt-4
30 (ALL), U397 (histiocytic lymphoma), SUP-T8 (ALL), U-373MG (glioblastoma), and T98G (glioblastoma).

Cell extracts prepared from enzyme-positive cells were electrophoresed on a 12.5% polyacrylamide gel containing 0.1% sodium dodecyl sulfate along with various
35 amounts of MTase which was purified from bovine liver as described above.

More particularly, the crude cell extracts (10-150 μ g/lane) were separated by electrophoresis in 12.5% polyacrylamide gels containing 0.1% sodium dodecyl sulfate. After electrotransfer to nitrocellulose membranes (0.45 mm; Bio-Rad, Richmond, CA), nonspecific binding sites were blocked with 3% powdered milk in BBS. The proteins were then probed for 16 h at room temperature with antisera diluted 1:500 in BBS containing 3% powdered milk. After the proteins were washed extensively with BBS, reactive bands were detected by the binding of 125 I-protein A (ICN Radiochemicals, Irvine, CA) for 1 hour. The membranes were washed and blotted onto paper towels and exposed to Kodak XAR-5 (tm) film at -70°C.

The bands on the autoradiographs were scanned with a densitometer (Bio-Rad) and were quantitated using a calibration curve obtained from the immunoreactive bands of the purified enzyme.

C. Results

In the non-lung cell lines and biopsies (i.e., in the gliomas), sixty-seven percent (4 of 6) were entirely deficient in immunoreactive enzyme (FIGURE 2). Six successive biopsy specimens from human gliomas, with different histological characteristics (Table I), five were entirely deficient (FIGURE 3). Control experiments showed that normal human brain has abundant MTase activity (FIGURE 3, lane 7). Thus, complete MTase deficiency is a common and specific metabolic abnormality in human gliomas.

Of 19 non-small cell lung cancer cell lines tested, MTase was entirely lacking in 6 cell lines (see, Table I and FIGURE 4).

EXAMPLE II

CLONING OF METase FROM *Psuedomonas putida*

Referring to the partial amino acid sequence for METase published by Wakayama, et al., *Biochem*, 27:1587-

1591, 1988), degenerate oligonucleotide primers were designed and used in a PCR assay for the gene for METase.

This PCR assay amplified a fragment of approximately 300 bp. The 300 bp PCR product was subcloned into the
5 plasmid pBluescript II KS (Stratagene, San Diego). Using an internal oligonucleotide probe to the PCR product, Southern blot analysis of this subcloned PCR product verified the identity of this fragment to be of the METase gene. Further Southern blot analysis showed that
10 this PCR generated fragment hybridized to a 5.0kb Bgl II fragment in *Pseudomonas putida* DNA.

Based on these results, a bacteriophage genomic DNA library was constructed containing *Psuedomonas putida* genomic DNA. Bgl II digested *Psuedomonas putida* was
15 electrophoresed on a 0.8% low melting point agarose gel. Bgl II fragments ranging in size of 4/kb to 6/kb were excised and purified from the gel. Using Klenow fragment, these Bgl II fragments were partially filled-in and subcloned into the bacteriophage vector, γ Fix II. This
20 vector was digested with Xho I and partially filled-in with Klenow. The library was packaged into bacteriophage particles using gigapack packaging extract from Stratagene. After packaging, the library was amplified and titered.

25 To isolate the complete METase gene, this library was screened using the PCR generated fragment. After screening 200,000 clones, eight independent primary clones were isolated. From these eight clones, only two clones were truly positive and unique. One clone
30 contained a 5.1kb insert and the other contained a 5.9kb insert. These inserts were subcloned into pBluescript II KS and were subsequently mapped and sequenced. We determined that the sequence for the METase gene was 1615 bp (see, SEQ.ID.No. 4).

35

EXAMPLE IIIEXPRESSION OF RECOMBINANT METase

The recombinant METase gene was expressed in the C5 vector. This is the same vector used for the expression of MTase (see, e.g., Example VII, co-pending U.S. Application No. 08/176,855, filed December 29, 1993). A single colony of C5 recombinant cloned *E. coli* was used to inoculate a 50 ml of culture. Standard LB medium was used supplemented with 50 μ g/ml ampicillin for both and large scale bacterial cultures. Inoculated 50 ml culture was incubated at 37°C overnight. The overnight culture was diluted 100-fold into fresh LB medium. Cells were grown in large culture (1l) for 1.5 hours with rigorous shaking at 37°C. To induce the METase expression, isopropylthio- β -D-galactoside [IPTG] was added at a final concentration of 0.01, 0.1, and 1 mM to the large culture and the cultures were incubated for an additional 4 hours. The optimum IPTG concentration for protein expression was found to be 1 mM.

Four hours following IPTG addition, the cells were collected and harvested by centrifugation at 19.000Xg 10 min. at 4°C. Supernatant was removed and pellet was suspended and washed in cold saline, then centrifuged again. The resuspended cell pellet was washed in 100-200 ml of 20 mM potassium phosphate buffer, pH 7.5, containing 15 μ M 2-mercaptoethanol. One mM-EDTA and 30 μ M-pyridoxal 5'-phosphate (buffer A) was added, then the pellet was spun again. The washed resuspended (in buffer) cell suspension was placed into cell disruption bomb. Cell breakage was done using 2.200 PSI N₂ pressure for 20 minutes. The lysed cells were centrifuged 43.000 x g for 20 min. at 4°C. The supernatant from the cell extract was further purified with dye-ligand affinity column.

Cell extract (10 ml) was placed onto a "DYEMATRIX" gel [Orange A] (Amicon Inc., Beverly, MA) column (12x2.6cm). The column was packed and equilibrated following the manufacturer's instructions. After the

sample loading the column was flushed with 5 column volumes of buffer A to remove unbound material. After this step, bound product was eluted with a 0-1.5 M KCl linear gradient in buffer A. Ten ml fractions were collected and subjected to the γ -lyase enzyme assay. The fractions containing the major peak of methionine γ -lyase activity were pooled and concentrated to 2-3 ml by "CENTRICON 30" (Amicon Inc.).

Solid $(\text{NH}_4)_2\text{SO}_4$ was added to the concentrated fractions (0.314 g/ml) to give a final concentration of 2.4 M, and the sample was centrifuged $13,000 \times g$ for 10 min. and supernatant was filtered with a 0.45μ acrodis filter (Amicon, Inc.) before injection onto an Alkyl "SUPEROSE" (agarose) Hr 5/5 hydrophobic-interaction-FPLC column (Pharmacia), that had been equilibrated with 2.4 M $(\text{NH}_4)_2\text{SO}_4$ dissolved in the buffer A used for previous steps. The bound protein was eluted by linearly decreasing the $(\text{NH}_4)_2\text{SO}_4$ concentration (flow rate 0.5 ml/min.). Fractions containing METase activity were pooled and concentrated as described earlier. The protein concentration was measured by the method described by Bradford.

The purity of enzyme preparation was checked by SDS 10% glycine-tris 1 mm gel (Novex, San Diego, CA). METase activity was assayed by measuring 2-ketobutyric acid production, as described by Esaki & Skoda (*Meth. Enzymol.* 143:459465 (1987), the disclosure of which is incorporated herein). The final enzyme had a specific activity of 300 U/mg where 1 U = 1 μ M product generated per minute.

EXAMPLE IV

SELECTIVE STARVATION OF MTase NEGATIVE CELLS IN NON-SMALL LUNG CANCER CELL LINES

The MTase negative non-small lung cancer cell lines identified in Example I were treated in vitro in a cell culture with METase and MTA according to the therapeutic method of the invention. Specifically,

enzyme-positive (SK-MES-1) and negative (A-549) cell lines were cultured for 4 days in (a) methionine-containing medium supplemented with 10% dialyzed horse serum, (b) methionine-depleted medium supplemented with 10% dialyzed horse serum, and (c) methionine-depleted medium supplemented with 10% dialyzed horse serum and 16 μ M MeSAdo. The proliferation of both cell lines, especially of the enzyme-negative A-549 cells, was markedly retarded in medium lacking methionine (27 and 3.3% growth of control for SK-MES-1 and A-549 cells, respectively). When MTA was added to the same medium, it augmented the growth of enzyme-positive SK-MES-1 cells (77% growth of control). However, the proliferation of enzyme-negative A-549 cells was not enhanced in the presence of MTA (4.3% growth of control) (Table II).

These data indicate that the growth of the MeSAdo phosphorylase-negative cells may be blocked selectively in methionine-depleted, MeSAdo-supplemented medium.

TABLE II

		<u>Growth (% of control)^b</u>		
		Methionine free		
	Cell Line	Enzyme Status ^a	Without MeSAdo	With MeSAdo
	SK-MES-1	+	27 \pm 2.6	77 \pm 4.7
	A-549	-	3.3 \pm 0.6	4.3 \pm 1.1

^a +, present; -, absent.

^b Percentage of control growth = 100 x (cell growth in methionine-depleted medium with or without MTA) / (cell growth in methionine-containing medium).

EXAMPLE VMETHIONINE STARVATION OF HUMAN MALIGNANT CELLS
WITH RECOMBINANT METase

To study the anti-proliferative effects of
5 recombinant METase produced as described in Examples II
and III, human SK-MES-1 and A-549 cells DMEM were
cultured in medium, and 10% dialyzed fetal bovine serum
supplemented with 0.06 U/ml recombinant METase. After
three days, cell proliferation was determined. The
10 effects of METase were expressed on a percentage of
cell growth in medium lacking added enzyme.

As shown in FIGURE 4, cell growth in the enzyme
positive (SK-MES-1) and enzyme negative (A-549) METase
supplemented medium increased, respectively, by 26.6
15 and 2.96%. However, if 20 μ MTA was added as an
alternate source of cellular methionine, cell growth
was restored to 61.4% of the control value in enzyme
positive cells, while growth in enzyme negative cells
declined to 2.0%.

20 SUMMARY OF SEQUENCES

SEQ.ID.No. 1 is the amino acid sequence for full-
length MTase.

SEQ.ID.No. 2 is the amino acid sequence of an
antigenic MTase peptide.

25 SEQ.ID.No. 3 is the amino acid sequence of an
antigenic MTase peptide which differs in amino acid
sequence from the peptide of SEQ.ID.No. 2.

SEQ.ID.No. 4 is the nucleotide sequence of a
polynucleotide encoding METase.

30 SEQ.ID.No. 5 is the amino acid sequence of METase
predicted from the nucleotide sequence of SEQ.ID.No. 4.

SEQUENCE LISTING

- (1) GENERAL INFORMATION:
- 5 (i) APPLICANT: THE REGENTS OF THE UNIVERSITY
OF CALIFORNIA
- 10 (ii) TITLE OF INVENTION: METHOD FOR SELECTIVE METHIONINE
STARVATION OF MALIGNANT CELLS IN MAMMALS
- (iii) NUMBER OF SEQUENCES: 5
- 15 (iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: Robbins, Berliner & Carson
(B) STREET: 201 N. Figueroa Street, 5th Floor
(C) CITY: Los Angeles
(D) STATE: California
20 (E) COUNTRY: USA
(F) ZIP: 90012
- (v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
25 (B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25
- (vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER: US
30 (B) FILING DATE:
(C) CLASSIFICATION:
- (vii) ATTORNEY/AGENT INFORMATION:
35 (A) NAME: Berliner, Robert
(B) REGISTRATION NUMBER: 20,121
(C) REFERENCE/DOCKET NUMBER: 5555-286
- (ix) TELECOMMUNICATION INFORMATION:
40 (A) TELEPHONE: 213-977-1001
(B) TELEFAX: 213-977-1003

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

- 5 (A) LENGTH: 2763 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

10

(vii) IMMEDIATE SOURCE:

- (B) CLONE: methyladenosine phosphatase

(ix) FEATURE:

15

- (A) NAME/KEY: CDS
(B) LOCATION: 1..2763

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

20

TTTATACAGA GCATGACAGT GGGGTCCTCA CTAGGGTCTG TCTGCCACTC TACATATTTG	60
AAACAGGAGT GGCTTCTCAG AATCCAGTGA ACCTAAATTT TAGTTTTAGT TGCTCACTGG	120
25 ACTGGGTTCT AGGAGACCCC CTGTGTTAGT CTGTGGTCAT TGCTAGSAGA ATCACTTAAT	180
TTTTTCTAGA CTCTAGGAGA AAACAGTTGG TGGTGACTC ATCACGGGTT AACAAATTCT	240
TCTCTCCTTC CATAGGCATG GAAGGCAGCA CACCATCATG CCTTCAAAGG TCAACTACCA	300
30 GGCGAACATC TGGGCTTTGA AGGAAGAGGG CTGTACACAT GTCATAGTGA CCACAGCTTG	360
TGGCTCCTTG AGGGAGGAGA TTCAGCCCGG CGATATTGTC ATTATTGATC AGTTCATTGA	420
35 CANNNNNNNN NNNNNNNNN GAGGTCGACG GTATCGATAA GCTTTGTAAA CAATTGTCTT	480
TAGCTTATCC AGAGGAATTG AGTCTGGAGT AAAGACCCAA ATATTGACCT AGATAAAGTT	540
GACTCACCAG CCCTCGGAGG ATGGAAGAT GGCCTTAAAA TAAACAAAC AAAAACCTTT	600
40 TTTGCTTTAT TTTGTAGGAC CACTATGAGA CCTCAGTCCT TCTATGATGG AAGTCATTCT	660
TGTGCCAGAG GAGTGTGCCA TATTCCAATG GCTGAGCCGT TTTGCCCCAA AACGAGAGAG	720
45 GTGTGTAGTC TTTCTGGAAG GTGTACCAGA ATAAATCATG TGGGCTTGGG GTGGCATCTG	780
GCATTTGGTT AATTGGCAGA CGGAGTGGCC CCATACCCTC ACTCAAGTTT GCTTTGTATT	840
ATGCAAGTTT ATGGAGAGTT ATTTCTGTG GCTAATAATT TNNNNNNNN NNNNNNNNN	900
50 AAGTGCAGCC TTAAGTTGTG CATGTGCTAG TATGTTTGA AGTTTCTGGT TTTTCTTTTC	960
TAGGTTCTTA TAGAGACTGC TAAGAAGCTA GGACTCCGT GCCACTCAA GGGGACAATG	1020
55 GTCACAATCG AGGGACCTCG TTTTAGCTCC CGGGCAGAAA GCTTCATGTT CCGCACCTGG	1080
GGGGCGGATG TTATCAACAT GACCACAGTT CCAGAGGTGG TTCTTGCTAA GGAGGCTGGA	1140
ATTTGTTACG CAAGTATCGC CATGGGCACA GATTATGACT GCTGGAAGGA GCACGAGGAA	1200
60 GCAGTAGGTG GAATTCTTTT CTAAGCACAT ATAGCATGGG TTTCTGGGTG CCAATAGGGT	1260
GTCTTAAC TG TTTGTTCTA TTACGTTAGT TTCAGAAAGT GCCTTTCTAC AAGGTTTGA	1320
65 AGTTGTTAAT ATTTTCTGTA GTTCCATTGG AAGGTAAGAA CAAAGATCAA AAGAAAGAAA	1380
GAGACACTTT TACCCAAGGA TCAGTAGTGA AAATAGTACA TTGTAGGCAT GTAGATGTGT	1440
TGAGAATCAT ACTAAGACTT GGGCCTTANN NNNNNNNNN NNNNNNNNN NNTACCCTAC	1500
70 ATTGAGGATT CGGTTTCAGC AGATAAATTT GAGGGACACA AACATTTAGG CTGTAGCAAG	1560
GCTGGAGCTC AGAAAAATGT TTTATGACAA GCAGTGAAT TTTAAGTTCT AGTAACCTCC	1620

28

AGTGCTATTG TTTCTCTAGG TTTCGGTGGA CCGGGTCTTA AAGACCCTGA AAGAAAACGC 1680
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5 AGAAACCCTC CATAACCTGA AGGTAAGTGC AGCCATGGAC AATCAGGCAT GTCTGTAGAC 1800
TCTCTATIGT CTTCCTTTCT TACTTGCACTT TCACCTTIGG TCCTCATGTA TTTTTTGCCA 1860
GCCTAGATGT TTTCAACAAG TTTTGTGAC ATCTACTACT ACCATACCAA CCACTTGTGA 1920
10 AACTGAGTAG TCTTATTTTC TTGGCTGGTA GTGCAGANNN NNNNNNNNNN NNAATAAACA 1980
ATAATCCAGG CTGGGCTGGT ATGGCAATAA GTGATTATCA GAACAATGCT CTGAGATAAG 2040
15 CATTATTAAC CTCACCTTAC AGGAAAGGGA GGTGAGGAAC CAAGAGITTA GAGTACCCGA 2100
AGTTCACAT CTGGTTAGTG AACTTGAAAA TTTTCTGTAG AATTTATTTA AAGTGTATGT 2160
TTCTGCGTC CTCACCTTGA TCTAGAAAAT CAAAATCTGT TTTTTTTTTT AACAAACATC 2220
20 TCAGTAATTA CGCCAACATG TGAATATCAC TGCCCTCTTT CTTCCTTTCA GAATATGGCC 2280
CAGTTTTCTG TTTTATTACC AAGACATTAA AGTAGCATGG CTGCCCAGGA GAAAAGAAGA 2340
25 CATTTCTAAT CCAGTCATTT TGGGAATTCC TGCTTAACTT GAAAAAATA TGGGAAAGAC 2400
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30 AGTAACATGT GGGAAAAAAT ATTACATTTT AAGGGGGAAA AAAAACCCCA CCATTCTCTT 2580
CTCCCCCTAT TAAATTTGCA ACAATAAAGG GTGGAGGGTA ATCTCTACTT TCCTATACTG 2640
35 CCAAAGAATG TGAGGAAGAA ATGGGACTCT TTGGTTATTT ATTGATGCGA CTGTAAATTG 2700
GTACAGTATT TCTGGAGGGC AATTTGGTAA AATGCATCA AAGACTTAAA AATACGGACG 2760
TAC 2763

40

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
45 (A) LENGTH: 17 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

50

(ii) MOLECULE TYPE: peptide

(vii) IMMEDIATE SOURCE:

(B) CLONE: methyladenosine phosphatase peptides

55

(ix) FEATURE:

(A) NAME/KEY: Peptide
(B) LOCATION: 1..17

60

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Ile Gly ile Ile Gly Gly Thr Gly Leu Asp Asp Pro Glu Ile Leu Glu
1 5 10 15

5 Gly

(2) INFORMATION FOR SEQ ID NO:3:

10 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 13 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

15 (ii) MOLECULE TYPE: peptide

(vii) IMMEDIATE SOURCE:
(B) CLONE: methyladenosine phosphatase peptides

20 (ix) FEATURE:
(A) NAME/KEY: Peptide
(B) LOCATION: 1..13

25 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

Leu Leu Leu Thr Thr Ile Pro Gln Ile Gly Ser Met Glu
1 5 10

30 (2) INFORMATION FOR SEQ ID NO:4:

35 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1615 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

40 (ii) MOLECULE TYPE: DNA (genomic)

(vii) IMMEDIATE SOURCE:
(B) CLONE: methionine-gamma-lyase

(ix) FEATURE:

(A) NAME/KEY: CDS

(B) LOCATION: 304..1497

5

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

10	ATAGGATGGC CTGGTAGCCA GTGATATAGC CGTTGCTTC CAGCAGCTTG ACCCGGCGCC	60
	AGCAGGGGCG AGGTGGTCAA TGCCACCTGG TCGGCAAGTT CGGCGACGGT TAGGCGGGCG	120
	TTGTCTGCA AGGCGGCGAG CAGGGCGCGG TCGGTGCGGT CGAGGCTTGA AGGCATGTTT	180
15	TGCCCTCTG GTCCGTTAAT TATTGTTTT GTTCCAGCAA GCACGCAGAT GCGTGGGCAA	240
	TTTTGAAAA AATCGGGCAG CTCGGTGGCA TAAGCTTATA ACAAACCACA AGAGGCTGTT	300
20	GCC ATG CGC GAC TCC CAT AAC AAC ACC GGT TTT TCC ACA CGG GCC ATT	348
	Met Arg Asp Ser His Asn Asn Thr Gly Phe Ser Thr Arg Ala Ile	
	1 5 10 15	
25	CAC CAC GGC TAC GAC CCG CTT TCC CAC GGT GGT GCC TTG GTG CCA CCG	396
	His His Gly Tyr Asp Pro Leu Ser His Gly Gly Ala Leu Val Pro Pro	
	20 25 30	
30	GTG TAC CAG ACC GCG ACC TAT GCC TTC CCG ACT GTC GAA TAC GGC GCT	444
	Val Tyr Gln Thr Ala Thr Tyr Ala Phe Pro Thr Val Glu Tyr Gly Ala	
	35 40 45	
35	GCG TGC TTC GCC GGG GAG GAG GCG GGG CAC TTC TAC AGC CGC ATC TCC	492
	Ala Cys Phe Ala Gly Glu Glu Ala Gly His Phe Tyr Ser Arg Ile Ser	
	50 55 60	
40	AAC CCC ACC CTG GCC TTG CTC GAG CAA CGC ATG GCC TCG TTG GAG GGT	540
	Asn Pro Thr Leu Ala Leu Leu Glu Gln Arg Met Ala Ser Leu Glu Gly	
	65 70 75	
45	GGT GAG GCG GGA TTG GCG CTG GCG TCG GGG ATG GGA GCC ATT ACT TCG	588
	Gly Glu Ala Gly Leu Ala Leu Ala Ser Gly Met Gly Ala Ile Thr Ser	
	80 85 90 95	
50	ACC CTC TGG ACC CTG CTG CCG CCT GGT GAT GAG CTG ATC GTG GGG CGC	636
	Thr Leu Trp Thr Leu Leu Arg Pro Gly Asp Glu Leu Ile Val Gly Arg	
	100 105 110	
55	ACC TTG TAT GGC TGC ACC TTT GCG TTC CTG CAC CAT GGC ATT GGC GAG	684
	Thr Leu Tyr Gly Cys Thr Phe Ala Phe Leu His His Gly Ile Gly Glu	
	115 120 125	
60	TTC GGG GTC AAG ATC CAC CAT GTC GAC CTT AAC GAT GCC AAG GCC CTG	732
	Phe Gly Val Lys Ile His His Val Asp Leu Asn Asp Ala Lys Ala Leu	
	130 135 140	
65	AAA GCG GCG ATC AAC AGC AAA ACG CGG ATG ATC TAC TTC GAA ACA CCG	780
	Lys Ala Ala Ile Asn Ser Lys Thr Arg Met Ile Tyr Phe Glu Thr Pro	
	145 150 155	
70	GCC AAC CCC AAC ATG CAA CTG GTG GAT ATA GCG GCG GTC GTC GAG GCA	828
	Ala Asn Pro Asn Met Gln Leu Val Asp Ile Ala Ala Val Val Glu Ala	
	160 165 170 175	
	GTG CCG GGG AGT GAT GTG CTT GTG GTG GTC GAC AAC ACC TAC TGC ACG	876
	Val Arg Gly Ser Asp Val Leu Val Val Val Asp Asn Thr Tyr Cys Thr	
	180 185 190	
	CCC TAC CTG CAG CCG CCA CTG GAA CTG GGG GCA GAC CTG GTG GTG CAT	924
	Pro Tyr Leu Gln Arg Pro Leu Glu Leu Gly Ala Asp Leu Val Val His	
	195 200 205	
	TCG GCA ACC AAG TAC CTC AGT GGC CAT GGC GAC ATC ACT GCG GGC CTG	972
	Ser Ala Thr Lys Tyr Leu Ser Gly His Gly Asp Ile Thr Ala Gly Leu	
	210 215 220	

31

	GTG GTG GGG CGC AAG GCT TTG GTC GAC CGC ATT CGG CTG GAA GGG CTG	1020
	Val Val Gly Arg Lys Ala Leu Val Asp Arg Ile Arg Leu Glu Gly Leu	
	225 230 235	
5	AAA GAC ATG ACC GGG GCA GCC TTG TCA CCG CAT GAC GCT GCG TTG TTG	1068
	Lys Asp Met Thr Gly Ala Ala Leu Ser Pro His Asp Ala Ala Leu Leu	
	240 245 250 255	
10	ATG CGC GGC ATC AAG ACC CTG GCG CTG CGC ATG GAC CGG CAT TGC GCC	1116
	Met Arg Gly Ile Lys Thr Leu Ala Leu Arg Met Asp Arg His Cys Ala	
	260 265 270	
15	AAC GCC CTG GAG GTC GCG CAG TTC CTG GCC GGG CAG CCC CAG GTG GAG	1164
	Asn Ala Leu Glu Val Ala Gln Phe Leu Ala Gly Gln Pro Gln Val Glu	
	275 280 285	

32

5 CTG ATC CAC TAC CCG GGC TTG CCG TCG TTT GCC CAG TAC GAA CTG GCA 1212
 Leu Ile His Tyr Pro Gly Leu Pro Ser Phe Ala Gln Tyr Glu Leu Ala
 290 295 300
 CAG CGG CAG ATG CGT TTG CCG GGC GGG ATG ATT GCC TTT GAG CTC AAG 1260
 Gln Arg Gln Met Arg Leu Pro Gly Gly Met Ile Ala Phe Glu Leu Lys
 305 310 315
 10 GGC GGT ATC GAG GCC GGG CGC GGC TTC ATG AAT GCC CTG CAG CTT TTT 1308
 Gly Gly Ile Glu Ala Gly Arg Gly Phe Met Asn Ala Leu Gln Leu Phe
 320 325 330 335
 15 GCC CGT GCG GTG AGC CTG GGG GAT GCC GAG TCG CTG GCA CAG CAC CCG 1356
 Ala Arg Ala Val Ser Leu Gly Asp Ala Glu Ser Leu Ala Gln His Pro
 340 345 350
 20 GCG AGC ATG ACG CAC TCC AGT TAC ACG CCA CAA GAG CGG GCG CAT CAC 1404
 Ala Ser Met Thr His Ser Ser Tyr Thr Pro Gln Glu Arg Ala His His
 355 360 365
 25 GGG ATA TCA GAG GGG CTG GTG AGG TTG TCA GTG GGG CTG GAG GAT GTG 1452
 Gly Ile Ser Glu Gly Leu Val Arg Leu Ser Val Gly Leu Glu Asp Val
 370 375 380
 GAG GAC CTG CTG GCA GAT ATC GAG TTG GCG TTG GAG GCG TGT GCA 1497
 Glu Asp Leu Leu Ala Asp Ile Glu Leu Ala Leu Glu Ala Cys Ala
 385 390 395
 30 TGAACCTGCC TTGCAGGATC GCGAACACTT GCCCAATGCC TCACGGGATC AGGCGATGGC 1557
 ACTTTGGATG AGCTGGTGAA TTGGCCGGCT TATCCAAGAG GAGTTTAAAA TGACCGTA 1615
 35 (2) INFORMATION FOR SEQ ID NO:5:
 (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 398 amino acids
 (B) TYPE: amino acid
 (D) TOPOLOGY: linear
 40 (ii) MOLECULE TYPE: protein
 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:
 45 Met Arg Asp Ser His Asn Asn Thr Gly Phe Ser Thr Arg Ala Ile His
 1 5 10 15
 50 His Gly Tyr Asp Pro Leu Ser His Gly Gly Ala Leu Val Pro Pro Val
 20 25 30
 Tyr Gln Thr Ala Thr Tyr Ala Phe Pro Thr Val Glu Tyr Gly Ala Ala
 35 40 45
 55 Cys Phe Ala Gly Glu Glu Ala Gly His Phe Tyr Ser Arg Ile Ser Asn
 50 55 60
 Pro Thr Leu Ala Leu Leu Glu Gln Arg Met Ala Ser Leu Glu Gly Gly
 65 70 75 80
 Glu Ala Gly Leu Ala Leu Ala Ser Gly Met Gly Ala Ile Thr Ser Thr
 85 90 95
 65 Leu Trp Thr Leu Leu Arg Pro Gly Asp Glu Leu Ile Val Gly Arg Thr
 100 105 110
 Leu Tyr Gly Cys Thr Phe Ala Phe Leu His His Gly Ile Gly Glu Phe
 115 120 125
 70 Gly Val Lys Ile His His Val Asp Leu Asn Asp Ala Lys Ala Leu Lys
 130 135 140
 Ala Ala Ile Asn Ser Lys Thr Arg Met Ile Tyr Phe Glu Thr Pro Ala
 145 150 155 160

33

Asn Pro Asn Met Gln Leu Val Asp Ile Ala Ala Val Val Glu Ala Val
 165 170 175
 5 Arg Gly Ser Asp Val Leu Val Val Val Asp Asn Thr Tyr Cys Thr Pro
 180 185 190
 Tyr Leu Gln Arg Pro Leu Glu Leu Gly Ala Asp Leu Val Val His Ser
 195 200 205
 10 Ala Thr Lys Tyr Leu Ser Gly His Gly Asp Ile Thr Ala Gly Leu Val
 210 215 220
 Val Gly Arg Lys Ala Leu Val Asp Arg Ile Arg Leu Glu Gly Leu Lys
 225 230 235 240
 15 Asp Met Thr Gly Ala Ala Leu Ser Pro His Asp Ala Ala Leu Leu Met
 245 250 255
 20 Arg Gly Ile Lys Thr Leu Ala Leu Arg Met Asp Arg His Cys Ala Asn
 260 265 270
 Ala Leu Glu Val Ala Gln Phe Leu Ala Gly Gln Pro Gln Val Glu Leu
 275 280 285
 25 Ile His Tyr Pro Gly Leu Pro Ser Phe Ala Gln Tyr Glu Leu Ala Gln
 290 295 300
 Arg Gln Met Arg Leu Pro Gly Gly Met Ile Ala Phe Glu Leu Lys Gly
 305 310 315 320
 30 Gly Ile Glu Ala Gly Arg Gly Phe Met Asn Ala Leu Gln Leu Phe Ala
 325 330 335
 35 Arg Ala Val Ser Leu Gly Asp Ala Glu Ser Leu Ala Gln His Pro Ala
 340 345 350
 Ser Met Thr His Ser Ser Tyr Thr Pro Gln Glu Arg Ala His His Gly
 355 360 365
 40 Ile Ser Glu Gly Leu Val Arg Leu Ser Val Gly Leu Glu Asp Val Glu
 370 375 380
 Asp Leu Leu Ala Asp Ile Glu Leu Ala Leu Glu Ala Cys Ala
 385 390 395
 45

CLAIMS

1. A method for the selective methionine starvation of cells in a mammal which are suspected of being MTase negative comprising:
 - 5 determining whether the cells are substantially MTase negative using means for detecting the presence or absence of both catalytically active and catalytically inactive MTase in a sample of the cells,
 - 10 administering a therapeutically effective amount of METase to the mammal, and at substantially the same time, administering a therapeutically effective amount of MTA to the mammal, wherein the METase and MTA are
 - 15 each administered in a pharmaceutically acceptable carrier.
2. A method according to Claim 1 wherein the METase is a recombinant microbial protein which will specifically
20 degrade mammalian methionine *in vivo*.
3. A method according to Claim 2 wherein the METase has substantially the same amino acid sequence as shown in
25 SEQ ID No. 5.
4. A method according to Claim 2 wherein the METase is expressed by a polynucleotide having substantially the same nucleotide sequence as shown in SEQ ID No. 4.
- 30 5. A method according to Claim 1 wherein the METase is coupled to polyethylene glycol.
6. A method according to Claim 1 wherein the METase and MTA are administered to the mammal at the same time.
35

21. The polynucleotide of Claim 18 having substantially the same nucleotide sequence as shown in SEQ.ID.No. 4.
- 5 22. A catalytically active METase polypeptide expressed by the polynucleotide of Claim 21.
23. The catalytically active METase of Claim 22 wherein expression of the polynucleotide is in a prokaryote.
- 10

1/4

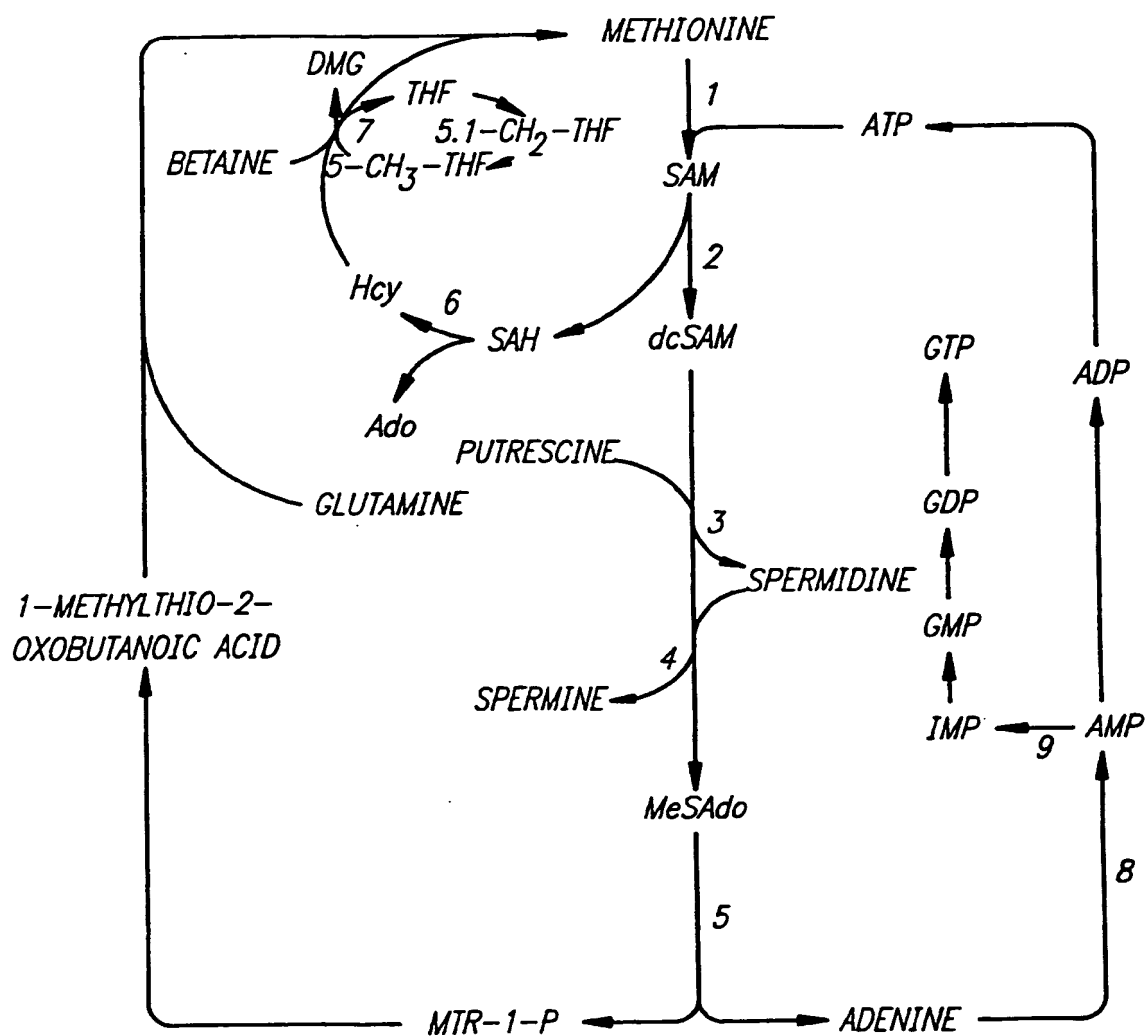



FIG. 1

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7. A method according to Claim 6 wherein the METase and MTA are mixed together in the same pharmaceutically acceptable carrier.
- 5 8. A method according to Claim 1 wherein the means for detecting the presence or absence of catalytically active and catalytically inactive MTase comprises an immunoassay.
- 10 9. A method according to Claim 1 wherein the means for detecting the presence or absence of catalytically active and catalytically inactive MTase comprises an assay including the following steps:
- 15 (a) obtaining an assayable sample of cells which are suspected of being MTase negative,
- (b) adding oligonucleotide probes which will specifically hybridize to a nucleic acid which will encode for MTase to the sample under conditions which will allow the probes to detectably hybridize to any such nucleic acid present in the sample, and
- 20 (c) detecting whether the nucleic acid is present in the sample.
- 25 10. A method according to Claim 9 wherein the sample is further subjected to conditions which will favor the selective amplification of any MTase encoding nucleic acid present in the sample.
- 30 11. A method according to Claim 1 further comprising the step of determining the mammal's plasma methionine level prior to and after administration to the mammal of the METase.

12. A method according to Claim 11 wherein the therapeutically effective amount of METase is between 10 units/m² and 20,000 units/m² administered at least once in a total amount sufficient to reduce the number of MTase negative cells detected in the mammal.
13. A method according to Claim 12 wherein the therapeutically effective amount of METase is that amount which will reduce the mammal's plasma methionine levels to about $\leq 10\%$ of its level prior to administration of the METase.
14. A method according to Claim 1 wherein the therapeutically effective amount of MTA is that amount which will be sufficient to maintain a plasma MTA concentration in the mammal of about 1-10 μM .
15. A DNA molecule having the nucleotide sequence shown in SEQ ID No. 4.
16. A catalytically active recombinant METase polypeptide.
17. A polypeptide according to Claim 16 which has substantially the same amino acid sequence as shown in SEQ ID NO. 5.
18. A polynucleotide which encodes for a catalytically active METase polypeptide.
19. A catalytically active METase polypeptide expressed by the polynucleotide of Claim 18.
20. The catalytically active METase polypeptide of Claim 19 wherein expression of the polynucleotide is in a prokaryote.



MeSAdoase
MDBK
U937
CEM
R1.1 WT
L1210
COS
Vero

FIG. 2A



Molt-4
SUP-T8
BV173
Molt-16
U937
K-T1
NALL-1
K562
DHL-9
HSB2

FIG. 2B

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Primary Tumors

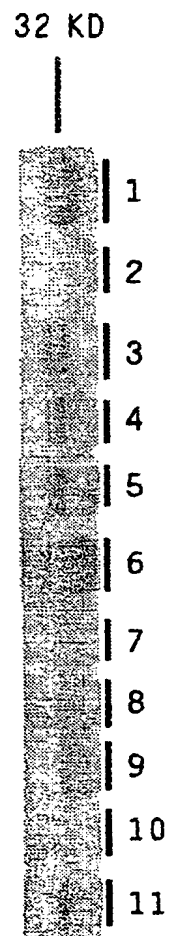


FIG. 3B

Cell Lines

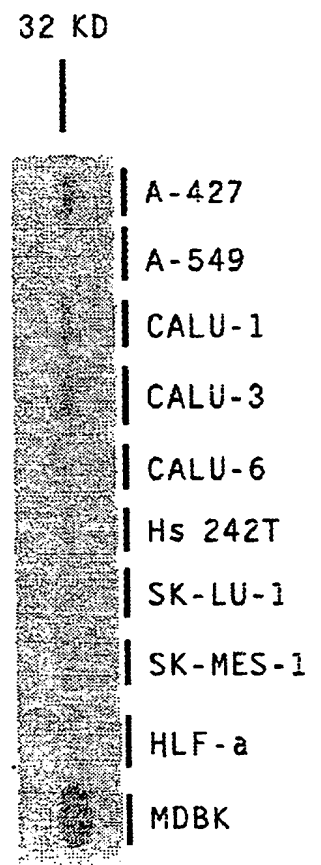


FIG. 3A

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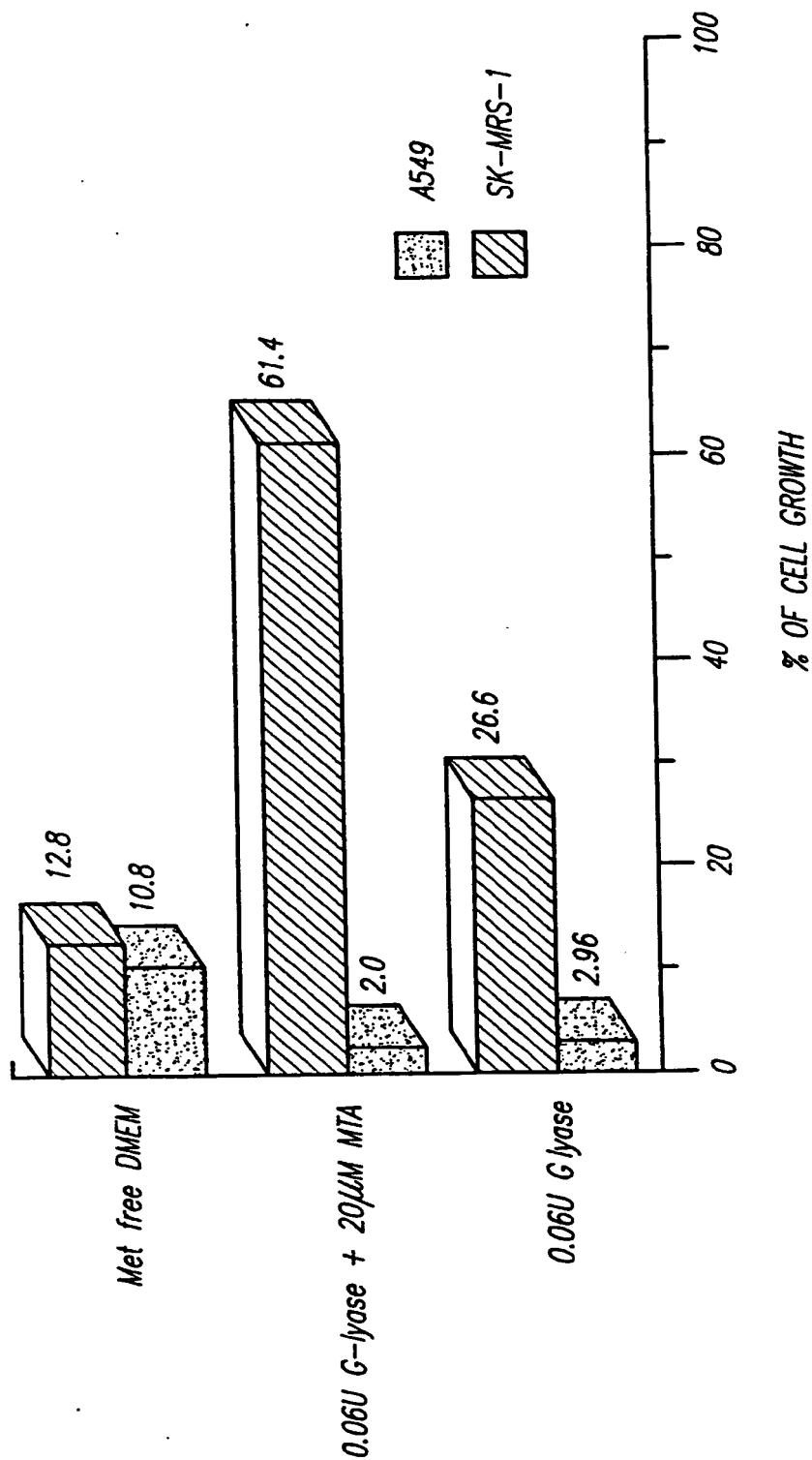


FIG. 4

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/14919

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : Please See Extra Sheet.

US CL : 424/94.3, 94.5; 514/46; 435/4, 6, 7.4, 232; 536/23.2

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/94.3, 94.5; 514/46; 435/4, 6, 7.4, 232; 536/23.2

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, BIOSIS, MEDLINE, LIFESCI, EMBASE, BIOTECHDS, CA, WPI

search terms: methionine or met, mtaase or methylthioadenosine phosphorylase, metase or methioninase or methionine(3a)lyase

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	BIOCHEMICAL PHARMACOLOGY, Volume 32, No. 19, issued 1983, M.J. Tisdale, "Methionine Synthesis From 5'-Methylthioadenosine by Tumour Cells", pages 2915-1920, see particularly page 2915 and 2920.	1-14
Y	ANNALS OF HEMATOLOGY, Volume 65 Supplement, issued 1992, J.S. Schwamborn et al., "Methylthioadenosine Phosphorylase Deficiency in Human Malignancy", page A121, abstract 359, see entire abstract.	1-14
Y	CANCER RESEARCH, Volume 53, issued 01 March 1993, T. Nobori et al., "Methylthioadenosine Phosphorylase Deficiency in Human Non-Small Cell Lung Cancers", pages 1098-1101, see entire document.	1-14

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:	* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
A document defining the general state of the art which is not considered to be of particular relevance	*X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
E earlier document published on or after the international filing date	*Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
L document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*G* document member of the same patent family
O document referring to an oral disclosure, use, exhibition or other means	
P document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search

17 FEBRUARY 1995

Date of mailing of the international search report

10 MAR 1995

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/14919

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	CANCER RESEARCH, Volume 51, No. 12, issued December 1991, T. Nobori et al., "Absence of Methylthioadenosine Phosphorylase in Human Gliomas", pages 3193-3197, see entire document.	1-14
Y	BIOCHEMICAL JOURNAL, Volume 281, issued 1992, D. Ragione et al., "Deficiency of 5'-Deoxy-5'-Methylthioadenosine Phosphorylase Activity in Malignancy", pages 533-538, see pages 534-536	1-14
X — Y	JOURNAL OF BIOCHEMISTRY, Volume 79, issued 1976, S. Ito et al., "Purification and Characterization of Methioninase From Pseudomonas putida", pages 1263-1272, see particularly pages 1265-1267.	16, 17, 19, 20, 22, 23 ----- 1-15, 18, 19, 21
X — Y	BIOCHEMISTRY, Volume 27, issued 1988, T. Nakayama et al., "Specific Labeling of the Essential Cysteine Residue of L-Methionine γ -Lyase with a Cofactor Analogue, N-(Bromoacetyl)pyridoxamine Phosphate", pages 1587-1591, see particularly pages 1588 and 1590.	16, 17, 19, 20, 22, 23 ----- 1-15, 18, 21
Y	CANCER RESEARCH, Volume 40, issued March 1980, W. Kreis et al., "Effect of Nutritional and Enzymatic Methionine Deprivation Upon Human Normal and Malignant Cells in Tissue Culture", pages 634-641, see entire document.	1-14
Y	ANTICANCER RESEARCH, Volume 13, issued 1993, V.K. Lishko et al., "Depletion of Serum Methionine by Methioninase in Mice", pages 1465-1468, see entire document.	1-14
Y	US, A, 5,122,614 (ZALIPSKY ET AL) 16 June 1992, see particularly columns 1-6.	5
Y	US, A, 4,179,337 (DAVIS ET AL) 18 December 1979, see particularly columns 1-7.	5

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/14919

A. CLASSIFICATION OF SUBJECT MATTER:

IPC (6):

A61K 38/51, 31/52, 47/48; C12N 9/88, 15/60; C12Q 1/00, 1/68; G01N 33/573

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